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CYTOTOXIC EFFECTS OF NEW GENERATION OXAZAPHOSPHORINES ON HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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The possible cytotoxic effects of three new generation oxazaphosphorines, D-17272 (mafosfamide cyclohexylamine salt), D-18864 (4-hydro-peroxy-cyclophosphamide) and D-19575 (β-D-glucose-isophosphoramide mustard), on human acute lymphoblastic leukemia MOLT-4 cells were studied. The influence of these agents on the leukemic cells was analyzed, using the in vitro spectrophotometric MTT test and Beckman Coulter method. The cell viability, as well as the cell size and count, were determined. The patterns of temporary changes in the analyzed parameters were dependent on the agent tested and its dose, and the time interval after the oxazaphosphorine application. The various MOLT-4 cell responses to the action of D-18864, D-17272 and D-19575 were shown.

Key words: new generation oxazaphosphorines, human acute lymphoblastic leukemia MOLT-4 cells, cell viability, cell size and count

INTRODUCTION

Oxazaphosphorines belong to alkylating chemotherapeutic drugs. The development of new oxazaphosphorine agents is of key importance to improve their therapeutic index. Several oxazaphosphorine derivatives have recently been synthesized and tested (BROCK and POHL, 2000; ENGEL et al., 2000; ZHANG et al., 2005a, 2005b; LIANG et al., 2007; GIRAUD et al., 2010; MAZUR et al., 2011).

The effects of the new generation oxazaphosphorines on hematopoietic cells have been investigated in preclinical studies (ASTA Medica, 1999; MAZUR et al., 2008a) and clinical trials (BRIASOULIS et al., 2000; VAN DEN BENT et al., 2003; GIACCONE et al., 2004; SHIMIZU et al., 2010). However, available information on the influence of oxazaphosphorines on human leukemic cells is still scarce (STYCZYŃSKI et al., 2002a, 2002b; MAZUR et al., 2009, 2010; OPYDO-CHANEK et al., 2010).

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The present study was undertaken to compare possible cytotoxic effects of three new generation oxazaphosphorine agents, D-17272 (mafosfamide cyclohexylamine salt), D-18864 (4-hydro-peroxy-cyclophosphamide) and D-19575 (β-D-glucose-isophosphoramide mustard), on human acute lymphoblastic leukemia MOLT-4 cells. The temporary changes in the leukemic cell viability, and in their size and count, were analyzed.

MATERIALS AND METHODS

Cells

Human acute lymphoblastic leukemia MOLT-4 cells (European Collection of Cell Culture, ECACC, Sigma Aldrich, Catalogue No. 85011413) were maintained in RPMI 1640 (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum (GIBCO BRL Life Technologies), 2 mM L-glutamine (Sigma Aldrich), and antibiotic antimycotic solution (Sigma Aldrich) containing 20 units of penicillin, 20 μg streptomycin and 0.05 μg amphotericin B. MOLT-4 cells were passaged every third day. The cells grew expotentially at 37°C in a 5% CO_2 atmosphere (HERAcell incubator, KendroLab). The cultures were periodically tested for *Mycoplasma* infection.

Chemicals

Mafosfamide cyclohexylamine salt (D-17272, CAS No. 84210-80-0), 4-hydro-peroxy-cyclophosphamide (D-18864, CAS No. 39800-16-3) and glufosfamide (D-19575, β-D-glucose-isophosphoramide mustard, CAS No. 132682-98-5) were obtained from NIOMECH (Bielefeld, Germany). D-17272, D-18864 and D-19575 were dissolved in aqua pro injectione (Polpharma). All the solutions were freshly prepared directly before treatment of the MOLT-4 cells.

Agent doses and cell treatment

After diluting the cell suspension to a density of $15x10^4$ cells/ml medium, MOLT-4 cells were subjected to 30-min exposure to the oxazaphosphorine agent. D-18864 was applied at a dose of 10 μg/ml medium, D-19575 at a dose of 100 μg/ml medium, and D-17272 at doses of 10 μg/ml and 100 μg/ml medium. The control material consisted of untreated MOLT-4 cells. After 30-min treatment with the oxazaphosphorine agent, MOLT-4 cells were centrifuged for 10 min at 1000 rpm and the supernatant was discarded. Then the cells were washed in 2 ml of PBS (BioMed) and pelleted by centrifugation for 7 min. The wash and centrifugation were repeated once more and the cells were resuspended in the complete RPMI 1640 medium.

Spectrophotometric MTT assay

The *in vitro* MTT test was designed for spectrophotometric determination of the viability of cell populations. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is the key component used in MTT assay. In viable, metabolically active cells, the tetrazolium ring is cleaved, yielding formazan crystals. Alterations in the metabolic activity of cell populations result in a concomitant change in the amount of formazan formed.

Protocol for *in vitro* MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich) was dissolved in RPMI 1640 medium, at a concentration of 5 mg/ml, and filtered through a 0.2 μm filter. 100 μl of the yellow MTT solution was added to each well of a 24-well plate, containing 1 ml of the cell suspension, and the cells were incubated at 37°C in 5% CO_2. Blank solution was prepared according to the above procedure using complete medium without cells. After the three-hour incubation period, the resulting formazan crystals were dissolved using 1 ml of acidified isopropanol (0.05 N HCl in absolute isopropanol). Absorbance of the obtained purple solution was measured at a wavelength of 570 nm using a Pharmacia Ultrospec III spectrophotometer (Pharmacia). The optical density of formazan solution was presented as the mean value ± standard deviation. The extent of MTT conversion in cells was also expressed as a percentage of the control value.

Cell size and count analysis

Samples of MOLT-4 cell suspension were taken from flasks and immediately diluted in ISOTON

II (Beckman Coulter filtered electrolyte solution based on 0.9 % saline). 500 µl of the cell suspension was added to 4.5 ml of ISOTON II. After the dilution of MOLT-4 cell suspension, individual leukemic cells were measured using a Z2 Coulter counter (Beckman Coulter, USA). The cell count and cell volume distributions for MOLT-4 cells were obtained using the counter equipped with a 100 µm diameter orifice. The flow rate was 500 µl / 12.5 sec. The range established for cell measurement was 65.4 – 3674 fl (5.0 – 19.1 µm). The cell count, diameter and volume were analyzed at 645.6 – 3674 fl (10.7 – 19.1 µm). The instrument was calibrated using 10 µm diameter latex beads (Beckman Coulter CC size standard). The mean and median cell volumes, modal peak and the mean cell diameter, as well as the cell count, were analyzed using Z2 AccuComp software (Beckman Coulter, USA). The cell count was presented as the mean value ± standard deviation.

Statistical evaluation

Statistical significance of alterations in the optical density of formazan and the cell count was evaluated by the analysis of variance and Duncan's new multiple range test.

RESULTS

The influence of the new generation oxazaphosphorines on the MOLT-4 cell viability (Table 1, Fig.1), the cell size (Table 2, Fig. 2) and the cell count (Table 3) was shown. The different patterns of temporary changes in the analyzed parameters were observed at 24h and 48h after the application of D-17272, D-18864 and D-19575. The effects of the oxazaphosphorines appeared to be dependent on the compound tested and its dose, and the time interval after the treatment of MOLT-4 cells with these alkylating agents (Table 1, 2, 3; Fig. 1, 2).

DISCUSSION

The results of the present study have demonstrated the different cytotoxic effects of the three oxazaphosphorines, D-17272, D-18864 and D-19575, on the human acute lymphoblastic leuke-

TABLE 1. The optical density of formazan solution determined in MOLT-4 cells following their exposure to the oxazaphosphorine agents. The metabolic activity and cell viability are correlated with the amount of formazan formed.

Experimental group			Time intervals after cell exposure to oxazaphosphorines	
			24h	48h
No.	Agent given	Agent dose	Optical density of formazan ($x10^{-3}$)	
			Mean ± SD	Mean ± SD
I	D-18864	10 µg/ml	2, 3, 4, 5, 48h 18.08 ± 0.90	2, 3, 4, 5, 24h 6.50 ± 0.55
II	D-17272	10 µg/ml	1, 3, 4, 5, 48h 29.58 ± 1.93	1, 3, 4, 5, 24h 15.17 ± 1.17
III	D-17272	100 µg/ml	1, 2, 4, 5, 48h 13.67 ± 2.88	1, 2, 4, 5, 24h 1.83 ± 0.41
IV	D-19575	100 µg/ml	1, 2, 3, 5, 48h 52.08 ± 3.80	1, 2, 3, 5, 24h 101.50 ± 2.58
V	Control		1, 2, 3, 4, 48h 55.92 ± 1.51	1, 2, 3, 4, 24h 116.42 ± 0.90

Statistically significant differences at $p<0.05$
Differences between groups: different from Group I – 1; Group II – 2; Group III – 3; Group IV – 4; Group V – 5.
Differences within each group: different from 24h – 24h; 48h – 48h; 72h – 72h.

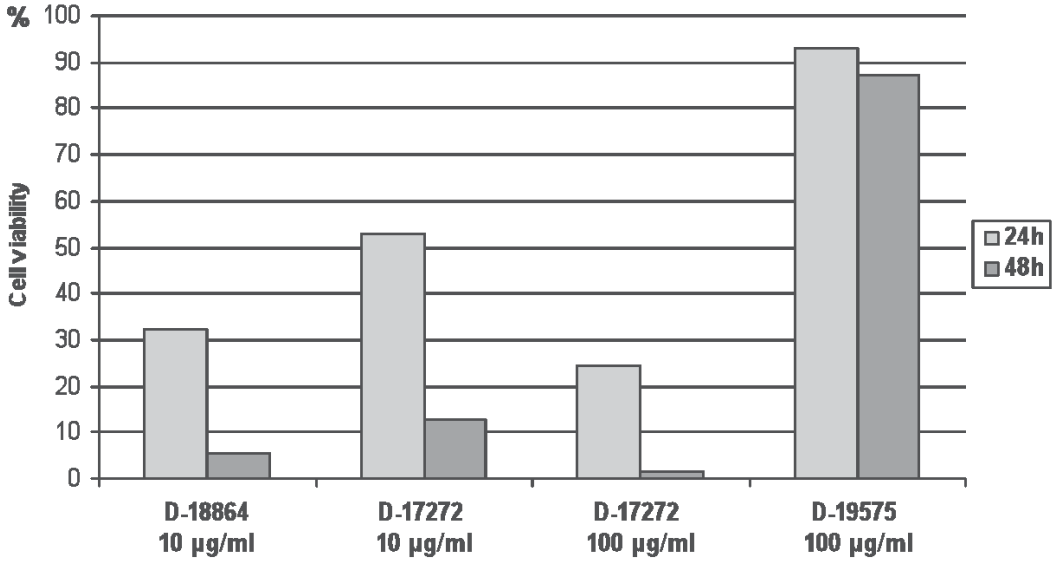

Fig. 1. Effects of the oxazaphosphorines on the MOLT-4 cell viability rate. The extent of MTT conversion in MOLT-4 cells expressed as a percentage of the control value.

TABLE 2. The size of MOLT-4 cells after their exposure to the oxazaphosphorine agents.

Experimental group			Time intervals	Mean cell volume [fl]	Median cell volume [fl]	Modal peak [fl]	Mean cell diameter [µm]
No.	Agent given	Agent dose					
I	D-18864	10 µg/ml	24h	1490	1442	1313	14.04
			48h	1275	1218	649	13.25
II	D-17272	10 µg/ml	24h	1640	1588	1499	14.48
			48h	1411	1355	649	13.71
III	D-17272	100 µg/ml	24h	1228	1158	653	13.11
			48h	1082	996	649	12.59
IV	D-19575	100 µg/ml	24h	1230	1172	1011	13.18
			48h	1307	1240	1085	13.44
V	Control		24h	1148	1089	954	12.88
			48h	1200	1139	970	13.06

mia MOLT-4 cells. It has been found that these alkylating agents distinctly affected the MOLT-4 cell viability as well as the cell size and count.

The three oxazaphosphorines caused a decrease in the metabolic activity of MOLT-4 cells, which manifested itself in the reduced cell viability rate.

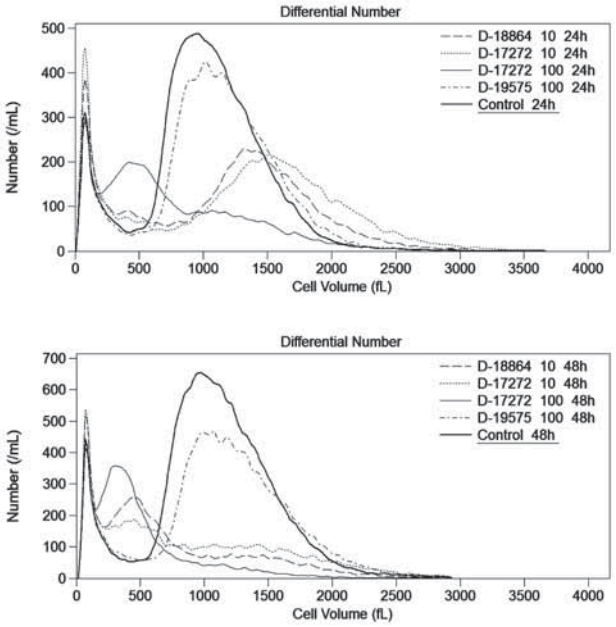

Fig. 2. The volume distribution curves for MOLT-4 cells measured using the Beckman Coulter counter. The peaks on the left represent cellular debris, presumably apoptotic bodies and cell fragments, which were excluded from the analysis of the MOLT-4 cell volume.

The functional changes occurring in MOLT-4 cells following their exposure to the oxazaphosphorines were surely responsible for the varied cell volume distribution. Moreover, the oxazaphosphorines influenced a reduction in the MOLT-4 cell count. These cytotoxic effects can result from metabolic disorders and loss of balance between the cell proliferation rate and cell death rate. In the previous investigations it has been observed that D-17272, D-18864 and D-19575 disturbed the cell cycle and cell proliferation rate as well as these alkylating agents induced mitotic catastrophe and triggered programmed cell death in leukemic cell lines (Mazur et al., 2002; 2008b, 2009, 2011; Opydo-Chanek, 2010).

D-19575 has been found to be less cytotoxic than D-17272, and especially than D-18864. The different cytotoxicity of the three oxazaphosphorines, observed in human leukemic MOLT-4 cells, is believed to be related to their varied action. The mechanisms responsible for the leukemic cell response to the action of the oxazaphosphorines have not yet been completely understood. However, their effects on cells are accepted to be dependent on the active alkylating moiety. Phosphoramide mustard and acrolein are the major active alkylating compounds of the two oxazaphosphorines, D-17272 and D-18864. D-19575 contains the directly alkylating moiety, isophosphoramide mustard. The metabolism of the oxazaphospho-

TABLE 3. The count of MOLT-4 cells after their exposure to the oxazaphosphorine agents.

Experimental group			Time intervals after cell exposure to oxazaphosphorines	
			24h	48h
No.	Agent given	Agent dose	Cell count (x10^3)	
			Mean ± SD	Mean ± SD
I	D-18864	10 μg/ml	2, 3, 4, 5, 48h 146.70 ± 5.94	2, 3, 4, 5, 24h 88.43 ± 1.94
II	D-17272	10 μg/ml	1, 3, 4, 5, 48h 166.94 ± 6.72	1, 3, 4, 5, 24h 126.73 ± 1.96
III	D-17272	100 μg/ml	1, 2, 4, 5, 48h 69.04 ± 2.59	1, 2, 4, 5, 24h 39.77 ± 1.27
IV	D-19575	100 μg/ml	1, 2, 3, 5, 48h 235.52 ± 11.15	1, 2, 3, 5, 24h 358.90 ± 5.44
V	Control		1, 2, 3, 4, 48h 258.88 ± 8.61	1, 2, 3, 4, 24h 458.99 ± 7.12

rines has a major impact on the pharmacodynamic-pharmacokinetic relationship, which can also influence the cell response to the action of these alkylating agents (Body and Yule, 2000; Engel et al., 2000; Zhang et al., 2005a; Liang et al., 2007; Mazur et al. 2011).

To summarize, it should be underlined that the metabolic activity based on the amount of formazan formed in the viable cells, as well as the cell size, are important parameters in determining and characterizing cytotoxicity of chemotherapeutic agents, including the novel oxazaphosphorine agents, such as D-17272, D-18864 and D-19575. The results of the present study should be taken into account in cancer therapy.

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